Abnormal Cornified Cell Envelope Formation in Mutilating Palmoplantar Keratoderma Unrelated to Epidermal Differentiation Complex

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Mutilating palmoplantar keratoderma represents a heterogeneous group of disorders, unified by characteristic mutilation of the fingers or toes, associated with palmoplantar keratoderma. Although loricrin gene mutations were recently reported in Vohwinkel's syndrome and erythrokeratoderma, the genetic basis of mutilating palmoplantar keratoderma is largely unexplored. We studied a family of non-Vohwinkel's syndrome, nonerythrokeratoderma mutilating palmoplantar keratoderma. The proband and his sister were similarly affected. Recessive inheritance was expected from the consanguineous family history. The patients had hyperkeratosis restricted to the palms and the soles. No other body sites were affected. Digital constriction was seen on all the fingers and the mutilation was severe on the distal interphalangeal region of several fingers. Histopathologically, hyperkeratosis without parakeratosis was seen in the lesional skin. Ultrastructural, immunohistochemical, and immunoelectron microscopic analyses revealed malformed cornified cell envelopes, the abnormal intracytoplasmic loricrin retention, and reduced deposition of loricrin to cornified cell envelopes. Involucrin and small proline-rich proteins 1 and 2 were normally distributed. Sequencing of the entire exons and exon-intron borders of loricrin gene of the patients excluded a mutation in loricrin DNA sequence. Linkage analysis excluded the possibility of causative mutation in the epidermal differentiation complex region of 1q21, including loricrin, involucrin, small proline-rich proteins, filaggrin, and trichohyalin. These data confirm the presence of non-Vohwinkel's syndrome mutilating palmoplantar keratoderma phenotype with abnormal loricrin cross-linking at the final stage of cornified cell envelope formation, which is caused by mutations outside the epidermal differentiation complex region. ***Key words: genodermatosis/keratinization/loricrin/mutilation. J Invest Dermatol 111:133–138, 1998***

One of the striking events during the process of the terminal differentiation in stratified squamous epithelia such as the epidermis is the formation of a 15 nm thick layer of protein on the inner surface of the cell periphery, termed the cornified cell envelope (CCE) (Hohl, 1990; Greenberg *et al*, 1991; Reichert *et al*, 1993). The CCE is now known to be assembled by the accumulation of several distinct proteins, including involucrin (Rice and Green, 1979; Eckert *et al*, 1993), cystatin *a* (previously known as keratolinin) (Zettergren *et al*, 1984; Kartasova *et al*, 1987; Takahashi *et al*, 1992), several small proline-rich proteins (SPRP) (Kartasova *et al*, 1987; Backendorf and Hohl, 1992; Gibbs *et al*, 1993), trichohyalin (Lee *et al*, 1993), loricrin (Mehrel *et al*, 1990; Hohl *et al*, 1991; Yoneda *et al*, 1992; Yoneda and Steinert, 1993), and possibly filaggrin (Richards *et al*, 1988; Steven and Steinert, 1994) and keratin intermediate filaments (Abernethy *et al*, 1977; Steven and Steinert, 1994). Among these molecules, loricrin is one of the major precursor proteins of CCE (Steinert and Marekov, 1995).

A clinically and genetically heterogeneous group of disorders, known collectively as the palmoplantar keratodermas (PPK), are unified by the phenotypic characteristic thickening of the skin over the palms and soles (Stevens *et al*, 1996). Although spectacular progress has been made in the molecular basis of inherited disorders of the skin in recent years, the genetic defects underlying the PPK are still largely unknown. Epidermolytic PPK is caused by mutations in keratin 9 (Fuchs and Green, 1980; Bonifas *et al*, 1994; Rothnagel *et al*, 1995; Navsaria *et al*, 1995), which is expressed exclusively in the skin of palms and soles. In contrast, less is known about the molecular pathology of nonepidermolytic PPK. There have been reports of linkage of the diffuse nonepidermolytic PPK to keratin gene clusters on both chromosome 17q (Rogaev *et al*, 1993) and chromosome 12q (Lind *et al*, 1994) and a mutation in the V1 domain of keratin 1 was also reported in a diffuse nonepidermolytic PPK (Kimonis *et al*, 1994). Keratin 16 mutations have been reported in focal nonepidermolytic PPK (Shamsher *et al*, 1995), and mutations in keratin 6, 16, and 17 in the related disorder, pachyonychia congenita (Bowden *et al*, 1995; McLean *et al*, 1995). There has only been one report of linkage of a PPK, which maps to chromosome 18q near the desmosomal cadherin gene cluster (Hennies *et al*, 1995). In spite of these reports, we cannot exclude possible candidate molecules for other phenotypes of PPK (Magro *et al*, 1997).

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Abbreviations: CCE, cornified cell envelope; EDC, epidermal differentiation complex; EK, erythrokeratoderma; MPPK, mutilating palmoplantar keratoderma; PPK, palmoplantar keratoderma; SPRP, small proline-rich protein; VS, Vohwinkel's syndrome.

Recently, loricrin gene mutations were reported as causative defects in two distinct types of PPK, Vohwinkel's syndrome (VS) associated with ichthyosis (Maestrini *et al*, 1996; Korge *et al*, 1997) and erythrokeratoderma (EK) (Ishida-Yamamoto *et al*, 1997). Mutilation of the fingers is a characteristic clinical feature shared by these two types of PPK. In this context, one can predict that other forms of PPK with mutilation might be due to alterations in CCE formation. These facts stimulated us to study CCE formation, especially loricrin, in a family of non-VS, non-EK mutilating PPK (MPPK) in order to elucidate whether a loricrin abnormality is a common cause of mutilating phenotype of PPK. The present ultrastructural, immunofluorescent, and immunoelectron microscopic observations and DNA analysis suggest that abnormal loricrin cross-linking in the formation of CCE also underlies the phenotype of non-VS, non-EK MPPK in which loricrin gene mutations are absent.

MATERIALS AND METHODS

Cases The proband, also referred to as case 1, was a 38 y old male of Japanese origin, who was the first child of consanguineous parents (**Fig 1*a***). His maternal grandmother and paternal grandmother are sisters. His sister (case 2) was similarly affected with MPPK. There is no other patient with MPPK in the family as far as we know. Case 1 presented with palmoplantar keratotic lesions that developed from his infancy (**Fig 2*a***), gradually progressed, and became stable in adolescence. He had no other skin abnormalities. His hair, teeth, and nails appeared normal. His hearing was normal and he had no other congenital anomalies. His palms and soles were hyperkeratotic and had a waxy appearance. Pseudoainhum was noted on the joints of his right fourth and fifth and left third fingers, and mild constrictions were rotated on the joints of other fingers (**Fig 2*a***). Surgical excision of the constricted fourth finger and skin graft on the DIP joint of the right middle finger was carried out about 10 y ago. Skin biopsies were obtained from an involved, constricted area of the second finger and an uninvolved area of the forearm area under local anesthesia after informed consent, and were processed for conventional light and electron microscopy, immunohistology, and immunoelectron microscopy.

Case 2, the 33 y old sister of the proband, had very similar lesions to case 1. She displayed well demarcated hyperkeratosis on the palms and the soles with constrictions on the DIP joints of the fingers. Her hair, teeth, and nails appeared normal. Her hearing was normal as well.

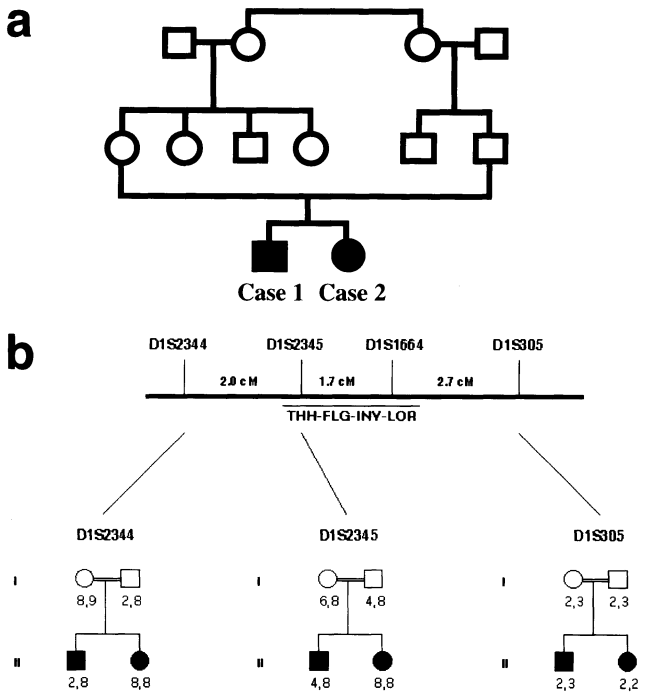

Figure 1. Linkage to the EDC on 1q21 is excluded in this family of MPPK. (*a*) Pedigree of the family. Case 1 is the proband. Note the consanguinity in the grandparents. (*b*) Analysis of microsatellite markers shows exclusion of linkage to the three microsatellites, D1S2344, D1S2345, and D1S305, in case 1 and case 2. Note that the proband and his sister have inherited different parental alleles for each of the three markers.

Antibodies The primary antibodies to CCE precursor proteins used in this study were rabbit polyclonal anti-human involucrin antibody (Biomedical Technologies, Stoughton, MA), mouse monoclonal anti-human SPRP 1 and 2 antibody (Kim *et al*, 1995), and rabbit polyclonal anti-human loricrin antibody (Mehrel *et al*, 1990).

Immunofluorescent labeling Six micrometer thick sections of fresh skin cut by cryostat were used as substrate. The sections were incubated in normal horse and goat sera for 30 min, and then incubated in primary antibody solution for 1 h at 37°C, followed by fluorescein isothiocyanate-conjugated to horse anti-mouse IgG and IgM (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Sections were extensively washed with phosphate-buffered saline between incubations. Counterstaining was done by incubating the sections in 10 μg propidium iodide per ml (to demonstrate nuclei) (Sigma, St. Louis, MO) for 10 s. Stained sections were mounted with a cover slip in mounting medium.

Post-embedding immunoelectron microscopy using cryofixation and cryosubstitution without chemical fixative Post-embedding immunoelectron microscopy using cryofixed and cryosubstituted skin specimens was carried out as described previously (Shimizu *et al*, 1989) with slight modification. Briefly, skin specimens were cryofixed by plunging them into liquid propane cooled to –190°C, followed by cryosubstitution; they were then embedded in Lowicryl K11 M (Chemische Werke Lowi, Waldkraiburg, Germany) at –60°C. The specimens were polymerized by ultraviolet irradiation. Ultrathin sections were incubated for 2 h at 37°C with a primary antibody. After being washed, each section was placed on a drop of 1 nm gold-labeled goat anti-mouse or rabbit IgG (Amersham International, Buckinghamshire, U.K.) diluted 1:40 at room temperature for 2 h, and was then washed with distilled water. For easier observation, the 1 nm gold particles were enlarged by incubation with immunogold silver-enhancement solution (Amersham International) at room temperature for 6 min (for observation at high magnification) or 10 min (for observation at low magnification) (Shimizu *et al*, 1992). The sections were counterstained with saturated uranyl acetate and lead citrate for 6 and 2 min, respectively.

Direct sequencing of the loricrin gene DNA was isolated from peripheral blood leukocytes of the proband (case 1), his sister (case 2), and their parents. The entire coding sequence of the loricrin gene was polymerase chain reaction (PCR) amplified from genomic DNA using the kit Expand Long Template PCR System with High Fidelity Taq (Boehringer, Mannheim, Germany), which alleviated the problems due to very high G-C content of the sequence.

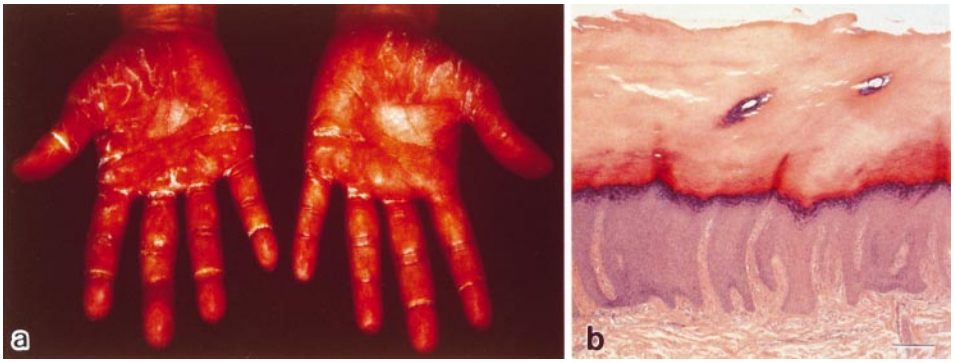

Figure 2. Characteristic clinical features and histopathology of the finger lesion of the proband. (*a*) The palm has a hyperkeratotic waxy appearance. Marked pseudoainhum is noted on the DIP joint of the right fourth and fifth and left third fingers. (*b*) Marked hyperkeratosis and moderate acanthosis are seen (hematoxylin and eosin stain). Hypergranulosis is present but parakeratosis is not seen. *Scale bar*, 100 μm.

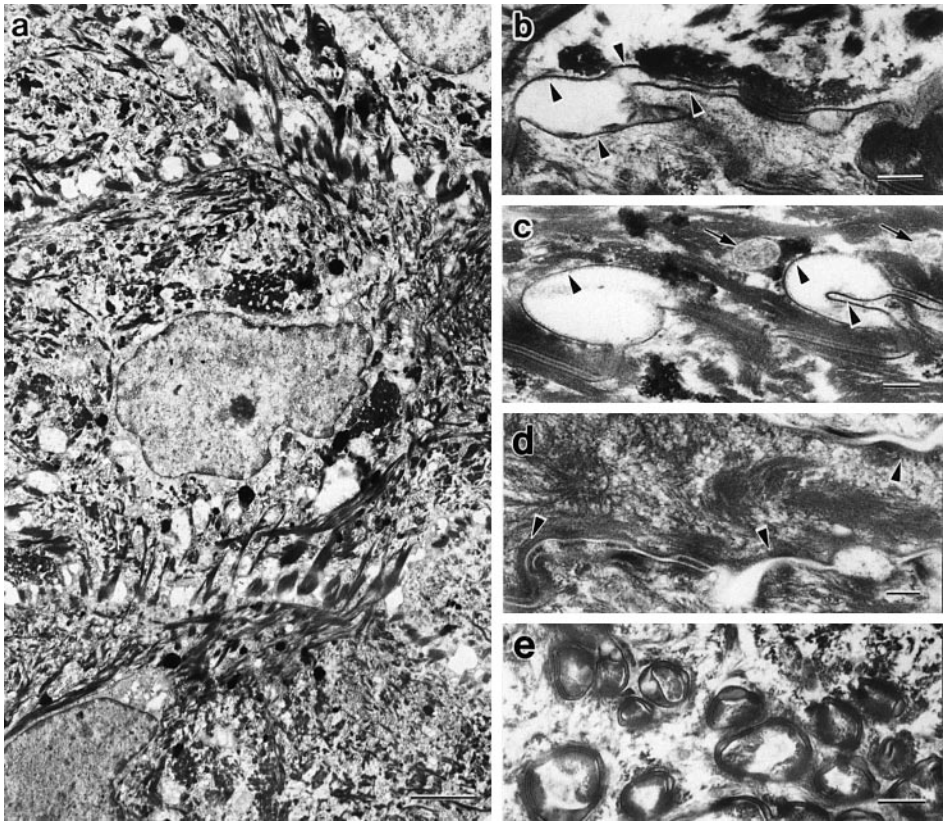

Figure 3. Electron microscopy of the lesional skin of the proband shows abnormal CCE formation. Granular cells have normal keratin bundles and keratohyalin granules (*a*). Thin CCE is normally formed in the granular cells (*b*), but in the cornified cells, CCE in patient skin (*c*) is remarkably thinner than in normal control skin (*d*). Ring-shaped structures of thin CCE and desmosomes are seen in an upper granular cell (*e*). *Arrowheads*, CCE; *arrows*, lipid droplets. *Scale bars*, (*a*) 2 μm, (*b–d*) 0.2 μm, (*e*) 0.5 μm.

The primers used to amplify exon 1 of loricrin were 5′-GTTTGACTCT-CTTAGGGCAC-3′ (forward) and 5′-GTCAGGCCTGGGCAAGACCA-3′ (reverse), derived from GenBank file M94077, and PCR was carried out at 95°C for 5 min, then 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The primers used to amplify exon 2, designed from the published sequence (GenBank M94077), were 5′-GCTGAGGCTCTGGCACCTG-AAAG-3′ (forward) and 5′-GCCGGAGAGCTCAATGGCTTCT-3′ (reverse), producing a PCR product of 1235 bp. PCR conditions were: 5 min at 95°C, then 1 min at 95°C, 45 s at 65°C, and 2 min at 72°C for 35 cycles. PCR products were verified on agarose gel, purified using the Centriflex Gel Filtration Column from ACGT, and directly sequenced, using the ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer-ABI, Foster City, CA). The sequences were run on an ABI 310 Prism Sequencer (Applied Biosystems, Foster City, CA).

Linkage analysis Microsatellite markers used for exclusion of the epidermal differentiation complex (EDC) region were part of the U.K. MRC Human Genome Mapping set (Reed *et al*, 1994). PCR reactions were performed using primers end-labeled with [γ^{33}P]dATP and PCR was carried out according to the following program: 95°C for 5 min, followed by 27 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and electrophoresis on a 6% acrylamide gel followed by autoradiography. Genotypers were assigned by visual inspection. The marker map used was D1S534–1.0-D1S422–2.2-AFM135yc3–0.1-D1S2345–3.0-D1S2346–1.0-AFMa357ze5–0.1-D1S305–5.4-SPTA1–6.8-D1S484, with distances in centimorgans (Maestrini *et al*, 1996). The order of markers and distances were based on the Généthon (Dib *et al*, 1996) and the CHLC (Murray *et al*, 1994) human linkage map.

RESULTS

Thickening of CCE during keratinization was disturbed in the lesional skin Light microscopically, the biopsy skin sample from an involved area of the finger of the proband showed marked hyperkeratosis, slightly thickened granular cell layer, and mild acanthosis without parakeratosis (**Fig 2*b***). Electron microscopically, basal and spinous cells in the epidermis did not show significant morphologic abnormalities. Keratohyalin granules in the thickened granular cell layers appeared normal (**Fig 3*a***). The transitional cell layer, which is only occasionally observed in normal skin, was frequently encountered between the granular and cornified cells. Thin, electron dense cell envelopes were seen in the superficial granular cells (**Fig 3*b***); however, the increase in thickness of cell envelopes in the horny layer that always occurs in normal skin was not observed (**Fig 3*c***). Lipid droplets and remnants of cellular organelles were present in the cornified cells (**Fig 3*c***). Ring-shaped structures made up of cell membrane with thin CCE and desmosomes were frequently observed in the cytoplasm of the upper granular cells, suggesting phagocytosis or invagination of cell membrane with abnormally formed CCE into the cytoplasm (**Fig 3*e***). Non-desmosomal areas of these ring-shaped structures were very sparsely labeled with loricrin and involucrin antibodies in immunoelectron microscopy (data not shown).

Defects of loricrin cross-linking were observed in the lesional skin Immunofluorescence study revealed an abnormal distribution of loricrin in lesional skin. Membranous loricrin staining was absent in the granular and cornified cells, and a weak cytoplasmic staining was observed in the granular cells (**Fig 4*a***). In the uninvolved site of the patient, loricrin staining was as strong as that in the normal control skin, and its membranous staining can be seen in the granular and upper spinous layers (**Fig 4*b***). In normal control skin, loricrin staining was observed only in the granular layer. In contrast, the expression of other CCE precursor molecules, involucrin, and SPRP 1 and 2, was stronger than that in the normal control skin, and their membranous staining were observed in the upper spinous layer cells as well as granular cells (**Fig 5**).

By immunoelectron microscopy, CCE were sparsely labeled with the loricrin antibody in the patient's skin (**Fig 6*a***), whereas intense labeling was seen in normal skin (**Fig 6*b***). In addition, immunoelectron microscopy revealed that involucrin labels on CCE in patients skin was rich compared with that in normal skin (**Fig 6*c***, ***d***).

Absence of causative mutations in the entire EDC region of 1q21, including the loricrin gene Direct sequencing of the loricrin gene of the genomic DNA from the proband (case 1), his affected sister (case 2), and their parents revealed no mutation in both exons and exon-intron borders of the loricrin gene. Assuming an autosomal recessive mode of inheritance, by linkage analysis of three microsatellite markers spanning in 1q21 (D1S2344, D1S2345, D1S305), using DNA samples isolated from the proband, his affected sister, and their parents, the entire EDC region of 1q21 was excluded (**Fig 1*b***).

DISCUSSION

Loricrin is the major protein component of the CCE (Hohl *et al*, 1991; Yoneda *et al*, 1992; Yoneda and Steinert, 1993; Steinert and Marekov, 1995), transcribed from a single gene within EDC on chromosome 1q21, where approximately 25 genes involved in epidermal differentiation are known to reside (Backendorf and Hohl, 1992; Volz *et al*, 1993; Gibbs *et al*, 1993; Mischke *et al*, 1996; Marenholz *et al*, 1996). Genes in the EDC region are thought to be under the control of a master regulator, or locus control region, similar to the globin genes (Townes and Behringer, 1990). In both VS with ichthyosis and EK, loricrin gene mutations are one bp insertion and result in a frameshift and delayed termination of transcription (Maestrini *et al*, 1996; Korge *et al*, 1997; Ishida-Yamamoto *et al*, 1997). The wild-type loricrin polypeptide is 315 amino acids in length (Yoneda *et al*, 1992), and these mutations replace the carboxy-terminal one-third of loricrin with missense amino acids, obliterating approximately one-third of the glutamine and lysine residues involved in transglutaminase-mediated cross-link formation. These highly deleterious mutations are speculated to impair the function of loricrin in a dominant-negative manner, by diminishing its flexibility (Steinert *et al*, 1991) and by impairing its ability to cross-link to itself and to other cornified envelope components (Maestrini *et al*, 1996; Korge *et al*, 1997; Ishida-Yamamoto *et al*, 1997).

Vohwinkel's syndrome shows palmoplantar hyperkeratosis with a honeycomb appearance, pseudoainhum, and ichthyotic lesions on other body sites (Vohwinkel, 1929; Gibbs and Frank, 1966; Camisa and Rossana, 1984; Camisa *et al*, 1988), whereas progressive symmetric EK, or simply EK, is a rare inherited disorder of epidermal cornification characterized by erythematous hyperkeratotic plaques (Hopsu-Havu and Peltonen, 1970). These nonmigratory, erythematous plaques develop shortly after birth, and are distributed symmetrically over the body, particularly on the extremities, hands, feet, the buttocks, and sometimes the face, together with PPK (Nir and Tanzer, 1978; Levi *et al*, 1982; Maldonado-Ruiz *et al*, 1982; Nazzaro and Blanchet-Bardon, 1986; Lamprecht *et al*, 1988; Kudsi and Naeyaert, 1990; MacFarlane *et al*, 1991; Kiesewetter *et al*, 1993; Niemi and Kanerva, 1993; Gray *et al*, 1996). Our cases clinically showed PPK with mutilation of fingers that is similar to that seen in VS and EK; however, they lack the honeycomb appearance of hyperkeratosis, deafness, symmetrical erythematous plaques on the body, and apparent family history of dominant inheritance. This family as well as VS and EK belong to a group of MPPK that consist of several other cornification disorders including Mal de Meleda, keratosis palmoplantaris striata, pityriasis rubra pilaris, congenital ectodermal defect, pachyonychia congenita, etc. (Gibbs and Frank, 1966).

Defective loricrin deposition in our family was similar to that of reported cases of VS and EK with loricrin gene mutations (Maestrini *et al*, 1996; Korge *et al*, 1997; Ishida-Yamamoto *et al*, 1997). These similarities suggested an involvement of loricrin abnormality in our case, too; however, in cases of VS with loricrin gene mutation, abnormal loricrin distribution was reported even in nonlesional sites (Korge *et al*, 1997). To the contrary, membranous loricrin staining in the nonlesional skin of the patient was as strong as that seen in normal control skin. In addition, intranuclear loricrin granules in the granular cells and the parakeratotic cornified cells, which were seen in VS and

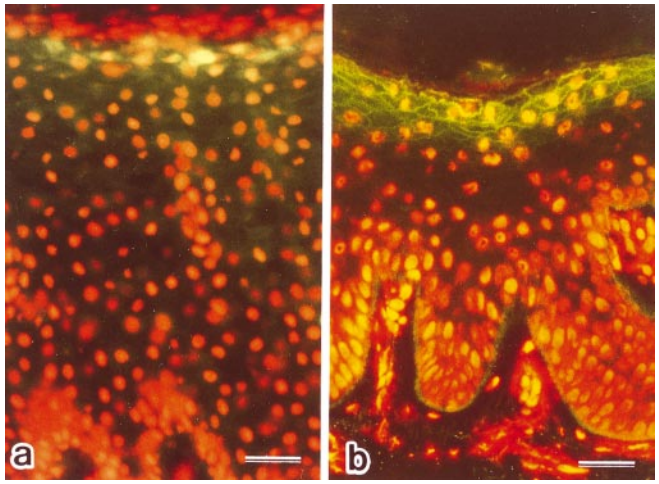

Figure 4. Weak cytoplasmic expression of loricrin in the granular layers of the lesional skin. Loricrin immunolabeling is detected by fluorescein isothiocyanate-conjugated secondary antibody (*green*) and nuclear stain is performed by propidium iodide (*red*). Loricrin immunolabeling in the lesional skin (*a*) and nonlesional skin (*b*) of the patient. Immunofluorescent labeling of loricrin is abnormally weak, cytoplasmic in the granular layers of the lesional skin in contrast to linear membranous stainings in the nonlesional site. *Scale bars*, 50 μm.

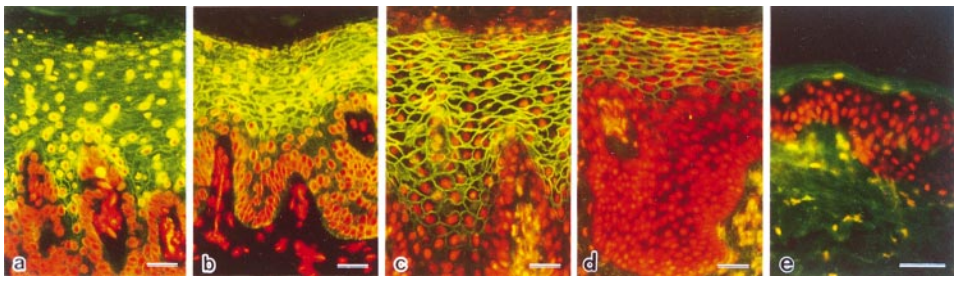

Figure 5. Membranous stainings in the granular and upper spinous layers both for involucrin and for SPRP 1 and 2 are similarly observed in lesional and nonlesional skin of the patient. Involucrin and SPRP 1 and 2 immunolabelings are detected by fluorescein isothiocyanate-conjugated secondary antibody (*green*) and nuclear stain is carried out by propidium iodide (*red*). Involucrin immunolabeling in lesional skin (*a*) and nonlesional skin (*b*), SPRP 1 and 2 immunolocalization in lesional skin (*c*) and nonlesional skin (*d*) of the patient and normal control skin (*e*). *Scale bars*, 50 μm.

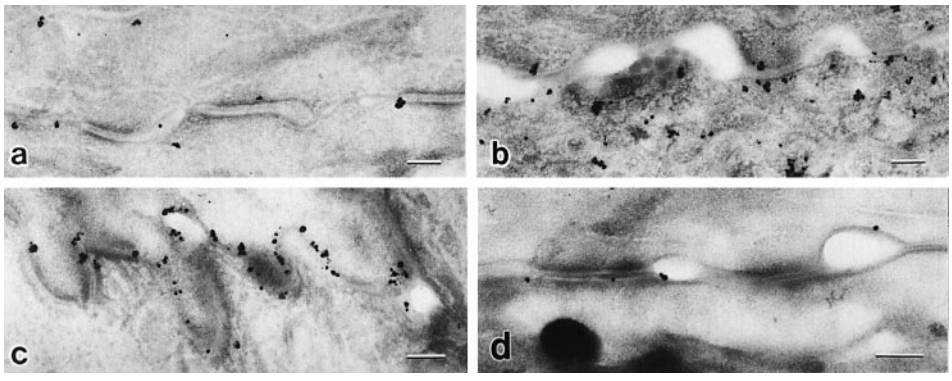

Figure 6. Post-embedding immunoelectron microscopy shows strikingly reduced loricrin deposition to CCE in lesional skin. Ultrastructural distribution of loricrin and involucrin is demonstrated by silver-enhanced immunogold particles. Note sparse loricrin labeling on CCE of a cornified cell in the patient skin (*a*), in contrast to dense loricrin labeling in normal control skin (*b*). On the other hand, involucrin labeling on CCE of a cornified cell is rich in the patient skin (*c*), but not in normal control skin (*d*). *Scale bars*, 0.2 μm.

EK with loricrin gene mutations (Maestrini *et al*, 1996; Korge *et al*, 1997; Ishida-Yamamoto *et al*, 1997), were not observed in our case. These results indicated that defective loricrin cross-linking is restricted to the lesional skin in our case and that the loricrin abnormality might be a secondary event. Furthermore, direct sequence analysis of the loricrin gene failed to detect any mutations in this family. Assuming an autosomal recessive mode of inheritance, linkage analysis excluded the possibility that the causative mutation is localized in the EDC region of 1q21 coding not only loricrin, but also SPRP, involucrin, trichohyalin, etc. (Backendorf and Hohl, 1992; Volz *et al*, 1993; Gibbs *et al*, 1993; Mischke *et al*, 1996; Marenholz *et al*, 1996). These data confirmed that a causative defect in this family is not in the loricrin or other EDC genes/molecules.

According to the consanguinity of the family and the absence of other affected members in the generations other than the proband and his sister, we can predict recessive inheritance of MPPK in this family. One can assume that the defect of loricrin cross-linking in this family is not due to a dominant negative effect reported in VS and EK with loricrin mutation (Maestrini *et al*, 1996; Korge *et al*, 1997; Ishida-Yamamoto *et al*, 1997), but by a total absence or defect of a key molecule for loricrin cross-linking. Loricrin cross-linking is thought to occur at the late stage of CCE formation (Steven *et al*, 1990; Bickenbach *et al*, 1995; Ishida-Yamamoto *et al*, 1996) and an unknown abnormality that can affect the final step of CCE formation is now suspected as the causative factor in this family.

In a family of VS without ichthyosis, a causative mutation outside the EDC region was suggested (Korge *et al*, 1997). It should be emphasized that the cases reported in this study are the first non-VS MPPK family in which mutations in the entire EDC region were excluded. Even if it is a secondary phenomenon, abnormal loricrin cross-linking may be a common event in several types of PPK with mutilation, including some families of VS, EK, and MPPK with or without loricrin gene abnormalities. Future investigations should serve to further define the molecular basis of each clinical phenotype of MPPK.

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